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Conservation of cysteine residues in fungal histidine acid phytases☆

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Abstract

Amino acid sequence analysis of fungal histidine acid phosphatases displaying phytase activity has revealed a conserved eight-cysteine motif. These conserved amino acids are not directly associated with catalytic function; rather they appear to be essential in the formation of disulfide bridges. Their role is seen as being similar to another eight-cysteine motif recently reported in the amino acid sequence of nearly 500 plant polypeptides. An additional disulfide bridge formed by two cysteines at the N-terminus of all the filamentous ascomycete phytases was also observed. Disulfide bridges are known to increase both stability and heat tolerance in proteins. It is therefore plausible that this extra disulfide bridge contributes to the higher stability found in phytase from some *Aspergillus* species. To engineer an enhanced phytase for the feed industry, it is imperative that the role of disulfide bridges be taken into cognizance and possibly be increased in number to further elevate stability in this enzyme.
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Keywords: Phytase; Cysteine; Disulfide bridge; Histidine acid phosphatase; BLAST; Bioinformatics; Enzyme stability

Interest in phytases has intensified over the last decade due to their established efficacy as a means to lower phosphorus levels in manure from swine and poultry production operations. The extensive use of soybean and other plant meals in the diet of these animals has created a need for phytase as a feed additive to enable these animals to effectively hydrolyze the phytic acid these meals contain. Supplementation of feedstuff with phytase has a dual benefit of increasing the bioavailability of the phytin phosphorus while decreasing the phosphorus levels in the manure. High phosphorus levels in animal manure in area of intensive production have the potential to create pollution problems in waterways [1].

A number of phytases have been extensively studied [2–6]. The majority of the characterized phytases share a compound catalytic mechanism and are members of a single class of enzymes, histidine acid phosphatases (HAPs) [7,8]. To distinguish these phytases from phytases featuring a different catalytic mechanism, the histidine acid phosphatases with high specific activity for phytic acid have been designated histidine acid phytases (HAPhy) [5]. The HAPhys from *Aspergillus niger* phyA [9], *Aspergillus fumigatus* phyA [10], and *A. niger* phyB [11] have been studied at an X-ray crystallographic resolution. Disulfide bonds are present in all these enzymes. In *A. niger* phyA they are important for maintaining the conformational stability and catalytic activity [12,13]. While 3-D molecular models of most phytases are not currently available, an analysis of the known amino acid sequence of other phytases reveals a unique pattern of conservation of cysteine residues necessary for the formation of these disulfide bridges, suggesting that disulfide bridges are an important component of HAPhys' structure. Even though these cysteine residues are not involved in catalytic mechanism of the molecule,

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they are conserved because of their essential role in the formation of disulfide bridges, which are necessary to achieve the proper molecular structure for the formation of both the active site and substrate-binding domain.

In this study, we have analyzed the deduced amino acid sequence of several fungal HAPhys deposited in the National Center for Biotechnology Information (www3.ncbi.nlm.nih.gov) specifically for conserved cysteine residues, which are obligatory for disulfide bridge formation. The cysteines associated with four of the disulfide bridge found in *A. niger* and *A. fumigatus* phyA were conserved in both the basidiomycete and the ascomycete HAPhys surveyed. Furthermore, a unique pair of cysteines for a N-terminal disulfide bridge is consistently found in the filamentous ascomycete HAPhys. A possible role for this extra disulfide bridge is also examined.

Methods

The ability of strains of *Saccharomyces cerevisiae* and other yeast isolates to hydrolyze phytate is widely known. While a 3-D structural model of HAPhys from non-filamentous fungi is not available at this time, the amino acid sequences of several yeast HAPs are accessible from various databanks. For this study, two *Schizosaccharomyes pombe* enzymes pho1 and pho2 previously listed as yeast phytases [2] and one *S. cerevisiae* acid phosphatase, pho3, shown to have phytase activity [14] were selected. In addition, the sequences of a *Debaryomyces occidentalis* phytase, identified in a patent [15], and a *Candida albicans* HAP were included. Quan et al. [16] have reported phytase in a *Candida* isolate. The active site motif characteristic of HAPs (RGHXRXY) is present in the amino acid (AA) sequence of all of these enzymes [7,8].

Several basidiomycete HAPhys have been cloned and sequenced [17]. Four of these isolates, *Peniophora lycii*, *Agrocybe pediades*, *Ceriporia* sp., and *Trametes pubescens*, were utilized in this study.

HAPhys from two filamentous ascomycetes, *A. niger* [9,11] and *A. fumigatus* [10], have been extensively studied and their crystal structures determined, and the genes from many other filamentous ascomycetes HAPhys phytases have been cloned and sequenced. The following filamentous ascomycete HAPhys were studied here; *Emericella nidulans* (anamorph: *Aspergillus nidulans*) Roche Nr. R1288, *Aspergillus terreus* 9A-1, *Thielavia heterothallica* (*Myceliophora thermophila* ATCC 48102), *Talaromyces thermophilus* ATCC 20186, *Penicillium oxalium* AAL55406, and *Neurospora sitophila* IFO 31635.

All the amino acid sequences utilized in this study were obtained from databanks maintained by the National Center for Biotechnology Information (NCBI) (www3.ncbi.nlm.nih.gov). Alignment of sequence and homology determinations were obtained from the NCBI's BLAST programs or by the Align Plus software program (Scientific & Educational Software, Durham, NC). The Genomic Disulfide Analysis Program (GDAP) was also employed to determine the possibility of disulfide bridges in phytase molecules not characterized by X-ray crystallography. The GDAP prediction server is accessed at www.doe-mbi.ucla.edu/Services/GDAP [18]. The NCBI's accession numbers for the macromolecular 3-D structure of *A. niger* phyA, *A. fumigatus* phyA, and *A. niger* phyB are 1IHP, 1QWO, and 1QFX, respectively.

Results and discussion

Aspergillus niger phyA and phyB are among the most thoroughly characterized fungal phytases. The 3-D molecular models of both have been described [9,11]. A pattern of conservation is observed in the distribution of their cysteine residues and disulfide bridge pairing. (Fig. 1). The cysteine residues for disulfide bridges 2, 3, and 5 in *A. niger* phyA (1, 3, and 5 in phyB) have maintained their relative positions, suggesting an essential role in the molecule's architecture.

Additional conserved cysteine motifs are also present in other fungal isolates. The percent homology between *A. niger* NRRL 3135 phyA, phyB, and the other isolates is presented in Fig. 2. The homology ranges from 23% to 66% for the complete sequences. However, for an eight-cysteine motif the conservation is 100%. In Fig. 3, a comparison of the sequence of *A. niger* NRRL 3135 phyA and representatives of non-filamentous ascomycete HAPs reveals a conservation of these eight cysteines known to form four disulfide bridges in *A. niger* phyA.

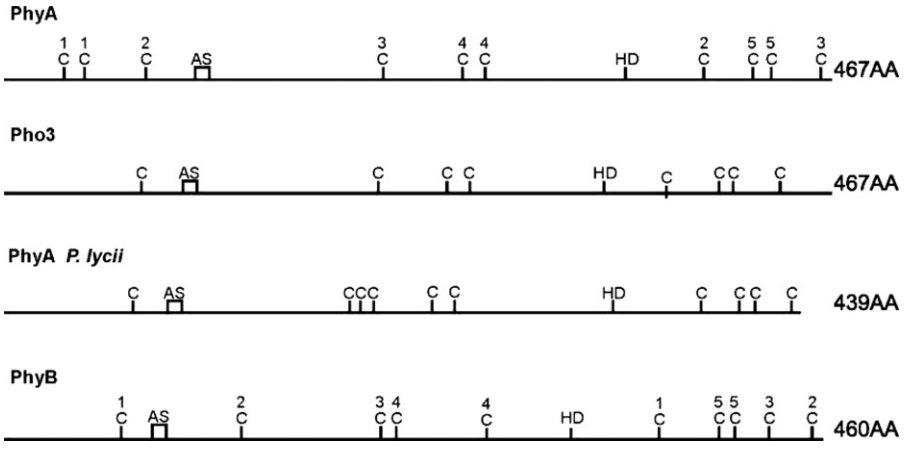

Fig. 1. Schematic representation of the cysteine distribution patterns in *A. niger* NRRL 3135, phyA, phyB, *S. cerevisiae* pho3, and *P. lycii* phyA. The number above each cysteine residue indicates the disulfide bond pairing if known. The position of the N-terminal active site motif is indicated by (AS) and its C-terminal component by (HD). AA indicates the number of amino acids in each sequence.

	phyA	phyB
Ascomycetes, Unicellular		
CA	28%	37%
DO	27	37
Pho1	25	28
Pho4	26	28
Pho3	27	33
Basidomycetes		
AP	36	28
CER	37	24
PL	39	25
TA	39	25
Ascomycetes, Filamentous		
AF	66	27
EM	61	26
AT	60	23
TH	48	24
TT	61	27
PO	60	23
NS	54	27

Fig. 2. The percentage of homology between *A. niger* NRRL 3135 phyA, phyB, and other fungal phytases. The NCBI locus number of the sequence and the unicellular ascomycetes are [CA] EAK94299, *C. albicans*, [DO] CAB70441, *D. occidentalis*, [Pho1] NP596847, *S. pombe* pho1, [Pho4] NP595181 *S. pombe* pho4, and [Pho3] CAA85045, *S. cerevisiae* pho3. The basidiomycetes are [AP] CAC48160 *A. pediades*, [CER] CAC48164 *Ceriporia* sp. CBS 100231, [PL] CAC48195 *P. lycii*, and [TA] CAC48234 *T. pubescens*. The filamentous ascomycetes are [AF] O00092 *A. fumigatus*, [EN] O00093 *E. nidulans*, [AT] O00085 *A. terreus*, [TH] AAB52508 *T. heterothallica*, [TT] AAB96873 *T. thermophilus*, [PO] AAL55406 *Penicillium oxalicum*, and [NS] BD188726 *N. sitophila*. The percent homology was determined by Align Plus software program.

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AN 10* ..(68)pagcrv..  (212)pgtctv..  (261)mdmcsf..
CA 8*  ..(59)pagcei..  (211)rsacsk..  (258)fswcay..
DO 8*  ..(59)paqcti..  (210)raacdn..  (258)faycay..
Pho1 9*..(55)pttcki..  (204)ynacpa..  (253)ygicsy..
Pho4 8*..(55)pescai..  (207)yyscpv..  (261)yyicvy..
Pho3 8*..(61)pegcem..  (212)gnacpg..  (260)fawcay..

AN    ..(279)spfcdlft..  (410)emmqcqa..  (432)plhgcpv..
CA    ..(271)spfcdlft..  (380)eklqcgn..  (400)pipkcat..
DO    ..(271)spfcdlft..  (380)ekyscgn..  (400)pipkcss..
Pho1  ..(266)sefcflfn..  (379)elfgced..  (400)plsdcgf..
Pho4  ..(274)sdfcslft..  (389)elflcsd..  (410)pltdcgy..
Pho3  ..(273)sdvcdift..  (384)ekfqcsn..  (404)pietcst..

AN    ..(442)grctrd..  (463)aecfa..    467 AA
CA    ..(410)fsckld..  (432)kqcgv..    461 AA
DO    ..(410)fscels..  (432)eqcdl..    461 AA
Pho1  ..(414)gmcely..  (440)tlcna..    463 AA
Pho4  ..(424)glcelc..  (450)sqcqa..    453 AA
Pho3  ..(414)fscein..  (437)kvcnv..    467 AA
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Fig. 3. The conservation of cysteine residues in *A. niger* NRRL 3135 phyA [AN] and non-filamentous ascomycete histidine acid phosphatase/phytases. The NCBI locus number of the sequence and organism are [CA] EAK94299, *C. albicans*, [DO] CAB70441, *D. occidentalis*, [Pho1] NP596847, *S. pombe* pho1, [Pho4] NP595181 *S. pombe* pho4, and [Pho3] CAA85045, *S. cerevisiae* pho3. The position of the first amino acid residue of each sequence fragment is indicated in parentheses. The * denotes the total number of cysteines in the sequence. AA indicates the number of amino acids in each sequence.

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AN 10* ..(68)pagcrv..  (213)gtctv..  (261)mdmcsf..
AP  9* ..(57)pegctv..  (194)nmcpn..  (238)mdmcpf..
CER 9* ..(56)pagcei..  (193)nmcpa..  (238)vslcpf..
PL 10* ..(55)pkdcki..  (192)amcpn..  (237)iplcaf..
TA 10* ..(60)pascqi..  (197)nmcpa..  (242)ltlcpf..

AN    ..(279)spfcdlft..  (410)emmqcqa..  (432)plhgcpv..
AP    ..(252)spfcdlft..  (379)eklacsg..  (399)plefcgg..
CER   ..(252)sdfctlfe..  (382)erlscag..  (402)plefcgg..
Pl    ..(251)spfcnlft..  (379)erllcqr..  (416)plkfcgg..
TA    ..(256)sefcdiye..  (385)erldcgg..  (405)plafcga..

AN    ..(442)grctrd..  (463)aecfa..    467 AA
AP    ..(408)gvcels..  (431)akcgf..    439 AA
CER   ..(412)glcald..  (435)ekcla..    442 AA
PL    ..(426)slctle..  (450)ekcfd..    453 AA
TA    ..(415)gvctld..  (438)ekcfa..    443 AA
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Fig. 4. The conservation of cysteine residues in *A. niger* NRRL 3135 phyA [AN] and basidiomycete HAPhys. The NCBI locus number and organism are [AP] CAC48160 *A. pediades*, [CER] CAC48164 *Ceriporia* sp. CBS 100231, [PL] CAC48195 *P. lycii*, and [TA] CAC48234 *T. pubescens*. The position of the first amino acid residue of each sequence fragment is indicated in parentheses. The * denotes the total number of cysteines in the sequence. AA indicates the number of amino acids in each sequence.

It is also interesting to note that while these ascomycete enzymes share a slightly higher percentage of homology with *A. niger* NRRL 3135 phyB (Fig. 2), the cysteines residues 263 and 276 in Pho3 (Fig. 1), and their respective cysteine residues in the other ascomycetes (Fig. 3) align closer to the configuration present in the phyA molecule.

There is also evidence to support this conserved pattern of four disulfide bridges in basidiomycetes. The deduced amino acid sequences of *P. lycii* and several other basidiomycete phytases are available, but the 3-D structural data are not. A comparison of the amino acid sequences with *A. niger* phyA (Figs. 1 and 4) reveals the conservation of the same eight-cysteine residues also found in the yeast HAPhys. Also, as in the non-filamentous ascomycete HAPhys, the two cysteines that form the N-terminal disulfide bridge in *A. niger* phyA are not present. In two of the basidiomycete HAPhys, *P. lycii* and *T. pubescens*, there are a total of 10 cysteines (Fig. 1). The locations of these additional residues vary in the two sequences and do not have homologous cysteines in either phyA or phyB.

The crystal structure of three filamentous ascomycetes HAPhys is known, however a 3-D molecular model is not available for other phytases cloned and sequenced

as possible candidates for use as an animal feed additive. This collection of phytase genes provides a bioinformatics resource to determine how widely the cysteine residues involved in disulfide bridge formation have been conserved. The alignment of HAPhys sequence from filamentous ascomycetes is given in Fig. 5. As shown in the previous figures, the cysteines necessary to form the four-disulfide bridges are all preserved. However, two additional cysteines associated with a fifth disulfide bridge in both *A. niger* and *A. fumigatus* phyA are present in the filamentous ascomycetes. It thus appears that the additional disulfide bridge is characteristic of HAPhys from this group. Further, the GDAP analysis of these sequences shows it is likely that these cysteines form disulfide bridges. In all the GDAP sequence comparisons, the highest homology is either with 1IHP or with 1QFX (data not shown).

The conservation of a conserved cysteine motif necessary for structural integrity is not a novel feature in phytases. An eight-cysteine motif has been reported in a group of approximate 500 plant polypeptides currently in various databases [19]. The motif is present in plant proteins having various functions and is seen as necessary for the maintenance of tertiary structure of these molecules. However, the cysteine motif found in these plant proteins does not resemble the phytase pattern In addition, neither the plant nor the fungal cysteine motifs have a catalytic function. It is likely that both

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AN 10*   ..(31)cdtvdggyqc.. (68)pagcrv..  (213)gtctv..
AF 10*   ..(30)cdtvdlgyqc.. (67)pkdcri..  (211)gvctk..
EN 10*   ..(29)cntadggyqc.. (66)phgcev..  (209)stcvs..
AT 11*   ..(31)cnsvdhgyqc.. (68)pegchi..  (213)slcta..
TH 10*   ..(26)cdtpdlgfqc.. (61)pddcev..  (206)dlcta..
TT 11*   ..(28)cntveggyqc.. (65)pqncki..  (210)gscpv..
PO 11*   ..(22)cdtvdggyqc.. (58)pekcel..  (203)ntcaa..
NS 10*   ..(27)cdspelgyqc.. (62)pegcrl..  (214)glcra..

AN       ..(261)mdmcsf.. (279)spfcdlft.. (410)emmqcqa..
AF       ..(259)mdmcsf.. (277)spfcqlft.. (408)etmqcks..
EN       ..(257)mdmcsf.. (275)spfcaift.. (406)elmqcek..
AT       ..(261)mamcpf.. (279)spfcdlft.. (410)emmqcra..
TH       ..(257)mdlcpf.. (286)spfcrlfs.. (417)ekmrcsg..
TT       ..(258)mdlcpf.. (275)spfcalst.. (406)emmqcdd..
PO       ..(251)mdmcay.. (269)spfcalft.. (401)emmqcgr..
NS       ..(263)mdlcpf.. (293)spfcslft.. (441)ekmicqs..

AN       ..(436)cpvdalgrc..   (463)aecf..    467 AA
AF       ..(434)cdvdklgrc..   (461)gecf..    465 AA
EN       ..(431)cavdkfgrc..   (458)ktcf..    463 AA
AT       ..(436)cptdklgrc..   (463)adcf..    466 AA
TH       ..(456)cgaderpmc..   (483)dlcf..    487 AA
TT       ..(432)cevdslgrc..   (459)egcy..    466 AA
PO       ..(427)cnvdqlgrc..   (454)akcg..    461 AA
NS       ..(479)ceadelgrc..   (506)dkcf..    510 AA
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Fig. 5. The conservation of cysteine residues in *A. Niger* NRRL 3135 phyA [AN] and other filamentous ascomycete HAPhys. The NCBI locus number and organism are [AF] O00092 *A. fumigatus*, [EN] O00093 *E. nidulans*, [AT] O00085 *A. terreus*, [TH] AAB52508 *T. heterothallica*, [TT] AAB96873 *T. thermophilus*, [PO] AAL55406 *Penicillium oxalicum*, and [NS] BD188726 *N. sitophila*. The position of the first amino acid residue of each sequence fragment is indicated in parentheses. The * denotes the total number of cysteines in the sequence. AA indicates the number of amino acids in each sequence.

function to provide the essential tertiary structure of the molecule, which is required to maintain a functioning active center. Additional studies of the fungal cysteine motif will become possible as more sequences are deposited in public databases.

It has not escaped our attention that a second *Aspergillus* HAPhys, phyB, also has five disulfide bonds. While it shares only 26% homology with *A. niger* phyA, the cysteines for three of the disulfide bridges are conserved (Fig. 1). The unique N-terminal disulfide bridge in phyA is not present in phyB, since this region stretches out to allow interfacing with its neighboring molecule to form a dimer. Unlike phyA, phyB is not a monomer [11]. However, the structural stability afforded by the additional disulfide bridges appears to be so essential that it also has two non-homologous disulfide bridges to maintain a total of five disulfide bridges. *A. niger* phyB is reported to have higher thermostability than phyA [20]. However, because of its polymeric structure when it is denatured at 90 °C, the protein cannot refold properly and is completely inactivated.

Phytases from filamentous ascomycetes surveyed for commercial application share some unique features [2]. More phytases have been characterized from this group than any other class of microorganisms. Their stability [21] and the heat tolerance [22] make them useful commercial enzymes. Disulfide bridges have been associated with both of these traits [23,24].

To date, all synthetic constructs to engineer an enhanced *Aspergillus* phytase [25] maintain the cysteines needed for the five-disulfide bridges. These studies report success in combining higher specific activity with an increase in thermal tolerance. In the *P. lycii* phytase, currently being marketed as an animal feed additive, Ronozyme P, lacks the N-terminal cysteines that form the fifth disulfide bridge of *A. niger* and *A. fumigatus* phyA (Fig. 1). A thermo-tolerant (CT) form of Ronozyme P (CT) is available, however its thermostability was achieved by formulation with a wax coating.

Phillippy [26] has recently reviewed a number of plant and microbial phytases for enzyme stability. There is considerable interest in identifying stable phytases and factors contributing to their enhanced stability. For example, Wyss et al. [27] have achieved this objective in *A. fumigatus* phytase by increasing its resistance to proteolytic degradation. Site-directed mutagenesis of the cloned *A. fumigatus* phy A gene was employed to introduce an amino acid sequence into a specific region of the enzyme to reduce its susceptibility to proteolysis. Introduction of additional disulfide bridges may offer another strategy for improving heat tolerance in this class of enzymes. Our study supports an essential role for the cysteine residues that are widely conserved in fungal HAPhys. As knowledge-based techniques are being employed to engineer an enhanced phytase, it is

important to further define the exact contributions disulfide bridge make to the stability of phytases. The possibility of achieving increased enzyme stability by additional disulfide bridges in the molecule also needs to be investigated.

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